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The GroEL Protein of the Whitefly *Bemisia tabaci* Interacts with the Coat Protein of Transmissible and Nontransmissible Begomoviruses in the Yeast Two-Hybrid System

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We have previously suggested that a GroEL homolog produced by the whitefly *Bemisia tabaci* endosymbiotic bacteria interacts in the insect hemolymph with particles of *Tomato yellow leaf curl virus* from Israel (TYLCV-Is), ensuring the safe circulative transmission of the virus. We have now addressed the question of whether the nontransmissibility of *Abutilon mosaic virus* from Israel (AbMV-Is) is related to a lack of association between GroEL and the virus coat protein (CP). Translocation analysis has shown that, whereas TYLCV-Is DNA is conspicuous in the digestive tract, hemolymph, and salivary glands of *B. tabaci* 8 h after acquisition feeding started, AbMV-Is DNA was detected only in the insect digestive tract, even after 96 h. To determine whether AbMV-Is particles were rapidly degraded in the hemolymph as a result of their inability to interact with GroEL, we have isolated a GroEL gene from *B. tabaci* and used a yeast two-hybrid assay to compare binding of the CP of TYLCV-Is and AbMV-Is to the insect GroEL. The yeast assay showed that the CPs of the two viruses are able to bind efficiently to GroEL. We therefore suggest that, although GroEL-CP interaction in the hemolymph is a necessary condition for circulative transmission, the nontransmissibility of AbMV-Is is not the result of lack of binding to GroEL in the *B. tabaci* hemolymph, but most likely results from an inability to cross the gut/hemolymph barrier.

Key Words: GroEL gene; circulative transmission; geminiviruses; whitefly.

INTRODUCTION

Geminiviruses are small plant viruses with circular single-stranded DNA (ssDNA) genomes encapsidated in ~20 × 30-nm geminate particles (Goodman, 1977; Harrison *et al.,* 1977). Begomoviruses are transmitted solely by the whitefly *Bemisia tabaci,* infect dicotyledonous plants, and possess either one (monopartite) or two (bipartite) genomic components. Monopartite begomoviruses have a genome ~2800 nucleotide (nt) long, encoding six functional open reading frames (ORFs). The genome of bipartite begomoviruses is split between two molecules of ~2600 nt each, DNA-A (basically similar to the genome of monopartite begomoviruses) and DNA-B encoding two ORFs (reviewed by Rybicki, 1994; Padidam *et al.,* 1995). In monopartite geminiviruses, the genomic molecule encodes the information required for replication, gene expression, particle assembly, and virus spread in plants. In bipartite geminiviruses, the functions for virus spread are encoded by DNA-B. *Tomato yellow leaf curl virus* (TYLCV) and *Abutilon mosaic virus* (AbMV) are members of the genus *Begomovirus.* Whereas AbMV has two genomic components, TYLCV isolates from around the Mediterranean have a single genomic component. On the other hand, TYLCV from Thailand has a bipartite genome (reviewed by Picó *et al.,* 1996; Czosnek and Laterrot, 1997; Nakhla and Maxwell, 1998).

The geminivirus coat protein (CP) dictates which insect transmits the virus. Exchanging the CP gene of the whitefly-transmitted *African cassava mosaic virus* (ACMV) with that of the leafhopper-transmitted *Beet curly top virus* (BCTV, genus *Curtovirus*) produced a leafhopper-transmissible ACMV-BCTV:CP chimera (Briddon *et al.,* 1990). Changes in the CP may lead to an inhibition of transmission. Mutating the CP gene of a bipartite begomovirus resulted in the loss of acquisition by *B. tabaci* (Azzam *et al.,* 1994). Replacing the CP of the nontransmissible AbMV with that of the transmissible *Sida golden mosaic virus* (SiGMV) produced a whitefly-transmissible chimeric AbMV (Höfer *et al.,* 1997). Two amino acid replacements in the CP of TYLCV from Sardinia, Italy (proline and histidine for glutamine and glutamine in positions 129 and 134), abolished virus transmission by *B. tabaci* but not its ability to systemically infect plants (Noris *et al.,* 1998).

The pathway of geminiviruses in the insect vector as well as the cellular and molecular processes underlying their transmission by whiteflies are progressively being unveiled. It is believed that virus particles are acquired along with phloem sap of infected host plants through the stylets and enter the esophagus and filter chamber (Harris *et al.,* 1995). Most likely, as observed for luteoviruses in aphids, virions are transported through the gut

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into the hemocoel. Virions that reach the salivary glands translocate into the salivary duct from which they are secreted with the saliva during feeding (Gildow and Gray, 1993; Gray, 1997). This path is completed by TYLCV from Israel (TYLCV-Is) in approximately 8 h (M. Ghanim, S. Morin, and H. Czosnek, 2000). Immunolocalization studies have suggested that the *B. tabaci* filter chamber and anterior portion of the midgut are possible sites involved in geminivirus transport from the gut lumen to the hemocoel (Hunter *et al.,* 1998).

Several begomoviruses, such as AbMV and *Honeysuckle yellow vein mosaic virus,* are ingested by the *B. tabaci* vector, are retained for several days, but are not transmitted by the insect (Bedford *et al.,* 1994; Wu *et al.,* 1996; Höfer *et al.,* 1997). The reason that these viruses are not transmitted by *B. tabaci* is not known. Several whitefly species, such as *Trialeurodes vaporariorum,* are able to ingest *B. tabaci*-transmissible begomoviruses but do not transmit them to plants (Cohen *et al.,* 1989; Polston *et al.,* 1990, Rosell *et al.,* 1999). The possible contribution of the whitefly digestive system and body fluid to discrimination between vector and nonvector insects has recently been studied. DNA of the transmissible begomovirus *Squash leaf curl virus* (SqLCV) could be detected by PCR in saliva and hemolymph of *B. tabaci,* but not in the saliva and hemolymph of the nonvector whitefly *T. vaporariorum,* despite virus ingestion by both, suggesting that the barrier to the transmission cycle occurs at the passage across the gut/hemocoel interface (Rosell *et al.,* 1999).

We have previously shown that a GroEL homolog produced by endosymbiotic bacteria of *B. tabaci* is implicated in the circulative transmission of TYLCV-Is. Feeding whiteflies with anti-*Buchnera* GroEL antiserum prior to acquisition of virions reduced TYLCV-Is transmission to tomato test plants by more than 80%. In the hemolymph of these whiteflies TYLCV-Is DNA was reduced to amounts below the threshold of detection by Southern blot hybridization. We have suggested that the viral particles that reach the hemolymph interact with GroEL on their way to the salivary glands, forming a complex that protects virions from rapid proteolysis (Morin *et al.,* 1999). This investigation together with that of Rosell *et al.* (1999) indicate that at least two different mechanisms may explain the nontransmissibility of begomoviruses: (1) the particles lose their ability to penetrate the insect gut epithelia; (2) the virions can reach the insect hemolymph but are unable to correctly associate with the GroEL protein. To determine whether either one or both mechanisms are responsible for the nontransmissibility of AbMV-Is by *B. tabaci,* we have first compared the paths of AbMV-Is and TYLCV-Is in *B. tabaci*'s organs and tissues implicated in the circulative transmission of begomoviruses. In a second step, we have cloned a GroEL gene from *B. tabaci* and studied the interactions between the CPs of AbMV-Is and TYLCV-Is and the *B. tabaci* GroEL protein, using a yeast two-hybrid assay.

RESULTS

An *Abutilon mosaic virus* isolate from Israel (AbMV-Is) is ingested but not transmitted by *B. tabaci*

Whiteflies were caged with AbMV-infected abutilon plants (*Abutilon selovianum*) for a 48-h access period. The insects were then collected and caged with 15 bean plants (*Phaseolus vulgaris,* cv. Tender green) for 21 days (20–50 insects per plant). None of the 15 bean plants exhibited disease symptoms or contained AbMV DNA (either DNA-A or DNA-B), detectable by Southern blot hybridization for as long as 3 months (not shown). It has to be noted that bean plants are susceptible to AbMV infection after bombardment with particles coated with cloned AbMV DNA-A and DNA-B from the West Indies ([AbMV-WI]; of the 9 plants treated, 6 were infected; data not shown).

Immunocapture-PCR experiments suggested that whiteflies that feed on AbMV-infected abutilon plants ingest virions. Following an access period of 4 days, about half the insect population was transferred to cotton plants, an AbMV nonhost. Extracts were prepared from groups of 20 whiteflies collected from both cotton and abutilon plants, 0, 3, 7, 10, and 14 days after the initial access to the infected abutilon plants. The extracts were incubated in PCR tubes coated with an antiserum raised against *Tomato golden mosaic virus* (TGMV) particles. The AbMV DNA, presumably associated with the CP bound to the antibody, was detected by PCR using DNA-A-specific primers. A DNA fragment of a similar size was also amplified from extracts of infected plants. AbMV DNA was not detected in nonviruliferous insects. It was not detected when PCR tubes were not coated with the antiserum or when the insect extract was omitted (Fig. 1). Viral DNA was found in whiteflies for up to 7 days after they have been transferred to cotton, but not after 10 or 14 days. In parallel, viral DNA was conspicuous in the insects that remained on the infected abutilon plants, indicating that the insects retained their capacity to ingest virus. These results indicated that AbMV DNA is present in *B. tabaci,* most likely encapsidated, for at least 7 days. Experiments using DNA extracted from whiteflies as PCR template indicated that AbMV DNA-A and DNA-B can be detected in the insect for up to 15 days after ingestion (not shown).

The nontransmissible AbMV-Is is found in the *B. tabaci* midgut, but not in the hemolymph and salivary glands of the insect

Figure 1 showed that AbMV-Is persists in whiteflies for at least 1 week after it has been ingested. Nonetheless

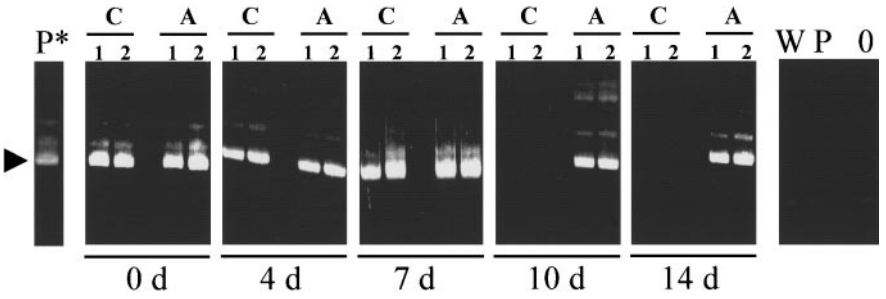


FIG. 1. Immunocapture-PCR detection of AbMV-Is DNA presumably associated with the virus CP in *B. tabaci.* Following a 4-day-long access to infected abutilon plants, about half the insects were transferred to cotton plants. Subsequently, two groups (1 and 2) of 20 whiteflies were collected from both cotton (C) and abutilon (A) plants at the time points indicated (0 to 14 days). The insect extracts were prepared for immunocapture-PCR analysis using PCR tubes coated with an antiserum raised against TGMV. Viral DNA was amplified using AbMV DNA-A-specific primers. The PCR products were subjected to agarose gel electrophoresis and stained. P*, extract of AbMV-infected plant; W and P, extracts of viruliferous whiteflies and infected plants, respectively, incubated in tubes not coated with the antiserum; 0, no extract; arrowhead, position of the ~800-bp amplified AbMV DNA fragment.

the virus is not transmitted by *B. tabaci.* Hence, the virus has to be blocked in one of the major organs participating in the circulative transmission of begomoviruses. Transmissible begomoviruses such as TYLCV-Is translocate from the stylets to the digestive tract, cross the midgut into the hemolymph, move to the salivary glands, and are finally inoculated with the saliva.

AbMV-Is was tracked by subjecting whitefly midgut, hemolymph, and salivary glands to PCR, following access periods on infected abutilon plants ranging from 0.5 h to 4 days. For each tissue, two groups containing pooled samples from three females were analyzed using DNA-A- and DNA-B-specific primers. The reaction products were separated on agarose gels, blotted, and hybridized with AbMV DNA-A- and DNA-B-specific probes. In a parallel experiment TYLCV-Is was similarly followed after acquisition access periods (AAP) on infected tomato plants. Figure 2 shows that after 8-h and 1- and 4-day access to infected abutilon plants, AbMV-Is DNA was detected in the whitefly midgut, but never in the hemolymph and the salivary glands. In comparison, TYLCV-Is DNA was conspicuous in the midgut, hemolymph, and salivary glands of insects that had access to the infected plants for 8 h and 1 and 4 days. These results suggest that, in contrast to TYLCV-Is, AbMV-Is is either unable to cross the gut/hemolymph barrier or is destroyed immediately after passing into the hemolymph.

The GroEL native protein is detected in *B. tabaci* hemolymph, but not in the insect digestive tract

Since AbMV-Is DNA was detected in the insect digestive tract for many days, we asked whether the virus was protected from digestive enzymes by GroEL, in a manner similar to that by which TYLCV-Is is protected in the hemolymph. The native, functional GroEL is an oligomer of 14 identical ~63-kDa subunits arranged in two stacked heptameric rings (Braig *et al.,* 1994). We have searched for the presence of native GroEL in the insect midgut and hemolymph. Protein extracts of midgut and hemolymph were subjected to nondenaturing polyacrylamide gel electrophoresis, Western blotted, and immunodetected with an antibody raised against aphid *Buchnera* GroEL (van den Heuvel *et al.,* 1994; Morin *et al.,* 1999). Partially purified *B. tabaci* GroEL served as marker (Morin *et al.,* 1999). Figure 3 (left panel) shows that a single band representing multimeric GroEL was observed in a partially purified *B. tabaci* GroEL preparation and in the insect hemolymph, but not in extracts of the digestive tract. SDS–polyacrylamide gel electrophoresis confirmed that a single ~63-kDa band corresponding to

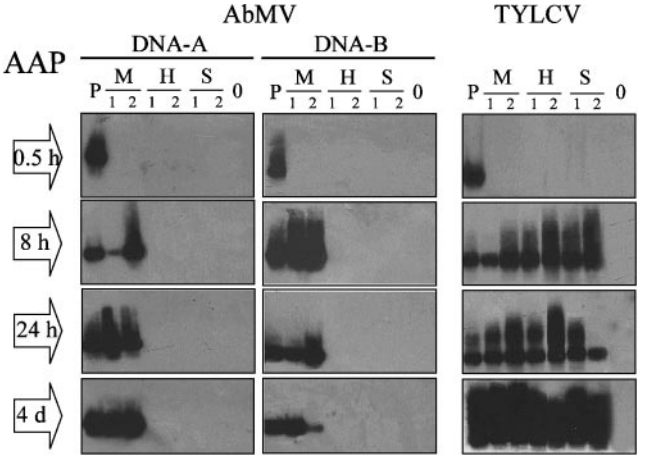


FIG. 2. Detection of TYLCV-Is and AbMV-Is DNA in *B. tabaci.* After the indicated access periods on AbMV-infected abutilon and on TYLCV-infected tomato plants, insect tissues involved in the circulative transmission of begomoviruses—midgut (M), hemolymph (H), and salivary glands (S)—were subjected to PCR. For each tissue, two sets (1 and 2) of pooled samples from three female insects were analyzed. DNA fragments of AbMV DNA-A and -B, and TYLCV were amplified using specific primers. The reaction products were subjected to agarose gel electrophoresis, blotted, and hybridized with AbMV DNA-A and -B, and TYLCV probes. PCR amplification was also done using an extract of an infected plant (P) and an extract of three nonviruliferous whiteflies (0).

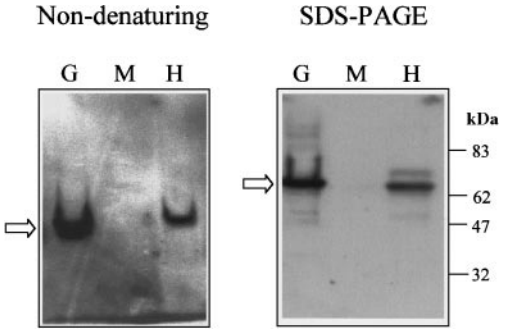


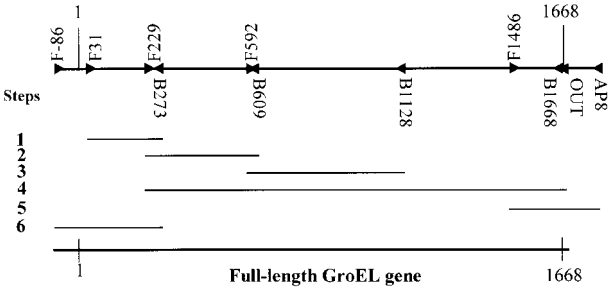
FIG. 3. Immunodetection of GroEL in *B. tabaci* tissues. Left panel: Western blot analysis using nondenaturing PAGE. Right panel: Western blot analysis using denaturing SDS–PAGE. G, partially purified native GroEL; M, midgut extract; H, hemolymph extract. Proteins were blotted and detected with anti-*Buchnera* GroEL IgG. Arrows: position of multimeric GroEL (left panel) and of the ~63-kDa GroEL monomer.

the GroEL subunit was immunodetected in the hemolymph but not in the digestive tract of *B. tabaci* (Fig. 3, right panel). These results demonstrate that GroEL cannot be detected in the insect digestive tract while it is present in the hemolymph of *B. tabaci* as a multimeric protein.

Cloning and sequencing of the GroEL gene homolog from *B. tabaci*

As a first step to study interactions between GroEL and begomoviral CP, we have cloned the *B. tabaci* GroEL gene. Overlapping fragments of the GroEL gene were amplified sequentially, starting from the 5′-end of the coding sequence and progressing toward the 3′-end of the gene. The strategy of amplification and the primers used are shown in Fig. 4. The first DNA fragment was amplified using a forward primer (F31), designed from the amino acid sequence of the protein N-terminus (Morin *et al.,* 1999), and on a backward primer (B273), based on a conserved GroEL gene sequence (Braig *et al.,* 1994; Fenton *et al.,* 1994). The amplified ~240-bp DNA fragment was sequenced and the information was used to design the 5′ primers that allowed amplification of downstream sequences. All subsequent 3′ primers were based on *Buchnera* (Hogenhout *et al.,* 1998) and *Escherichia coli* (Braig *et al.,* 1994; Fenton *et al.,* 1994) conserved regions of the GroEL gene. Four consecutive PCR amplification-sequencing steps were necessary to obtain the sequence of the full-length structural gene. Two additional reactions were necessary to obtain the 5′ and the 3′ flanking regions of the gene. The full-length GroEL gene from *B. tabaci* was amplified in one piece using primers F-86 and OUT, cloned and sequenced. The sequence of the full-length gene (GenBank accession number AF130421) was identical to that of the overlapping PCR fragments.

The *B. tabaci* B-biotype GroEL gene codes for 555 amino acids. It is a typical prokaryotic, intronless gene. To ascertain that *B. tabaci*-B GroEL has structural and functional similarities with other GroEL proteins, the deduced amino acid sequence of the *B. tabaci*-B GroEL gene was compared with that of representatives from the *Enterobacteriaceae* (*E. coli*, 85% similarity; GenBank accession number X07850), *Buchnera* (*Myzus persicae*, 84% similarity; GenBank accession number AF003957), γ-3 (*Pseudomonas aeruginosa*, 82% similarity; GenBank accession number U17072), β- (*Neisseria gonorrhoeae*, 79% similarity; GenBank accession number Z23008) and α-subdivisions (*Agrobacterium tumefaciens*, 75% similarity; GenBank accession number X68263). Figure 5 shows that all amino acids previously reported to be involved in polypeptide binding (indicated by arrows), GroES binding (indicated by arrowheads), and ATPase activity (indicated by asterisks) (Fenton *et al.,* 1994) were also found in *B. tabaci*-B GroEL. The only exception is the substitution of phenylalanine to tyrosine at position 44. Of the 133 amino acid residues (boxed in Fig. 5) previously reported to be conserved among 50 prokaryotic Hsp60/GroEL homologs (Braig *et al.,* 1994; Fenton *et al.,* 1994) all are shared by *B. tabaci*-B GroEL except for glycine at position 170 (Gly170) and glutamic acid at position 388 (Glu388). In *B. tabaci* Gly170 is replaced by threonine and Glu388 is replaced by alanine. Since the glycine to threonine substitution at position 170 is also found in the



Primer	Sequence (5' to 3')	Position on the gene
F-86	GACATT(T,C)T(A,G)GCAATTGTT	-86 to -69
F31	GA(C,T)GC(C,T)CG(A,T)AAAAAAATG	31 to 48
F229	GTCGCCTCTAAAGCAAACG	229 to 246
B273	(A,T)GT(G,A)GT(G,A)GTACCGTC(G,A)CCTGC	273 to 253
F592	GG(T,C)TATTT(A,G)TCTCCTTATTTT	592 to 612
B609	ATAAGGAGA(T,C)A(A,G)ATAACC	609 to 592
B1128	AAC(T,G)CC(T,G)CCTG(A,C)TAATTT(A,C)GC	1128 to 1108
F1486	GGAATTTTAGATCCTACTAAAG	1486 to 1507
B1668	TTACATCAT(T,G)CCGCCCAT	1668 to 1651
AP8	ACGACTCACTATAGGGCTTTTTTTTTTTTAA	+131 to +106
out	ATGTGTGTGATGTTTTTGG	+21 to +3

FIG. 4. Cloning the *B. tabaci* GroEL gene homolog. The strategy and the primers used to sequentially amplify GroEL DNA fragments and the entire gene are shown in the upper panel. The sequences of the primers are shown in the lower panel. Numbers refer to the *B. tabaci* GroEL gene coding sequence. Negative numbers indicate upstream sequences; positive numbers indicate downstream sequences.

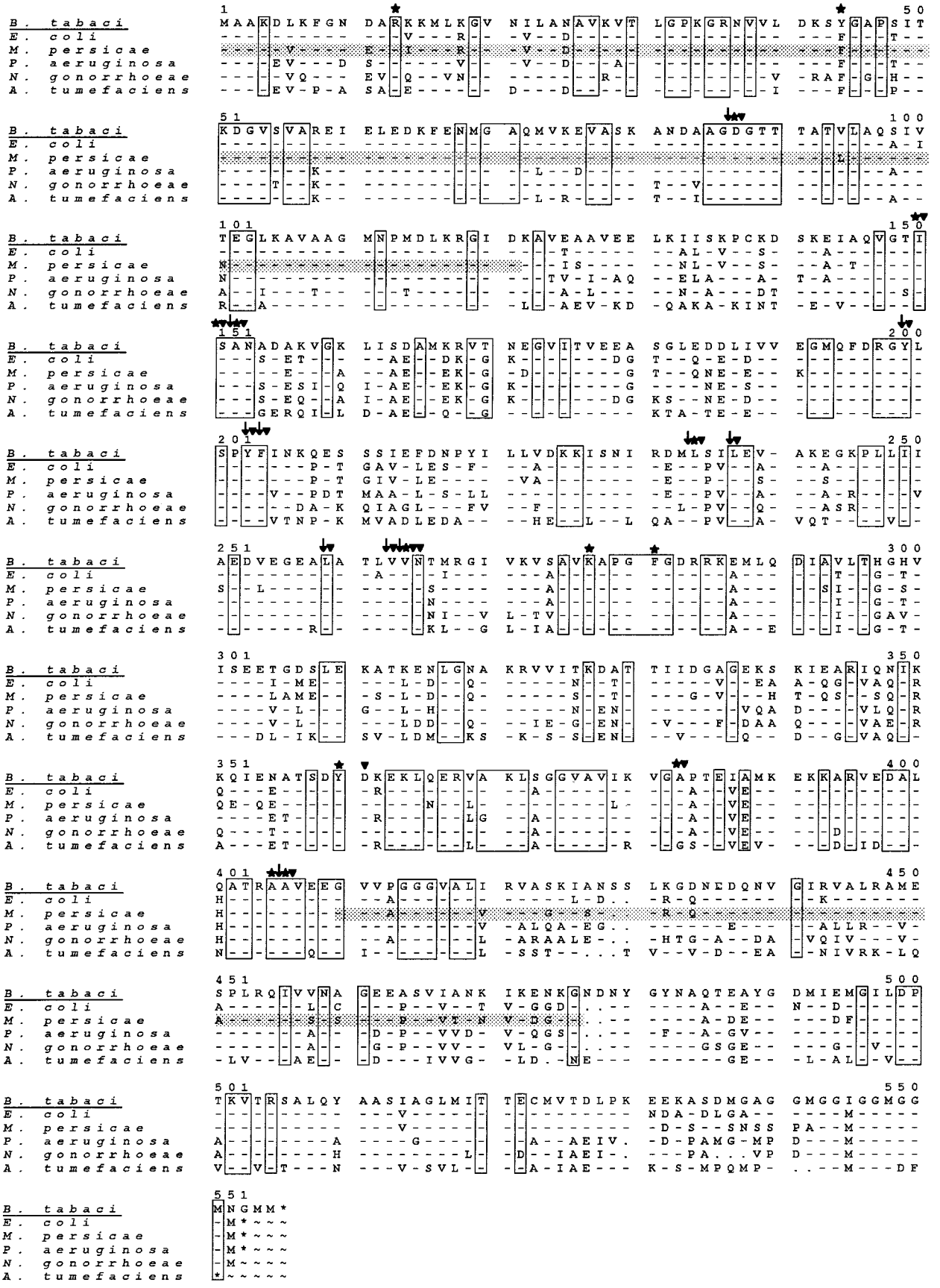


FIG. 5. Amino acid sequence of *B. tabaci* GroEL and comparison with GroEL from free living (*E. coli*, *P. aeruginosa*, *N. gonorrhoeae*, and *A. tumefaciens*) and endosymbiotic (*M. persicae*) bacteria. Identical amino acids are indicated by bars. Amino acids at positions conserved in other GroEL/Hsp60 chaperonins are boxed. Amino acids reported to be involved in polypeptide binding are indicated by arrows, those involved in GroES binding are indicated by arrowheads, and those involved in ATPase activity are indicated by asterisks (Fenton *et al.,* 1994; Braig *et al.,* 1994). The *Potato leafroll virus* (PLRV) binding domains (Hogenhout *et al.,* 1998) are indicated by gray shading.

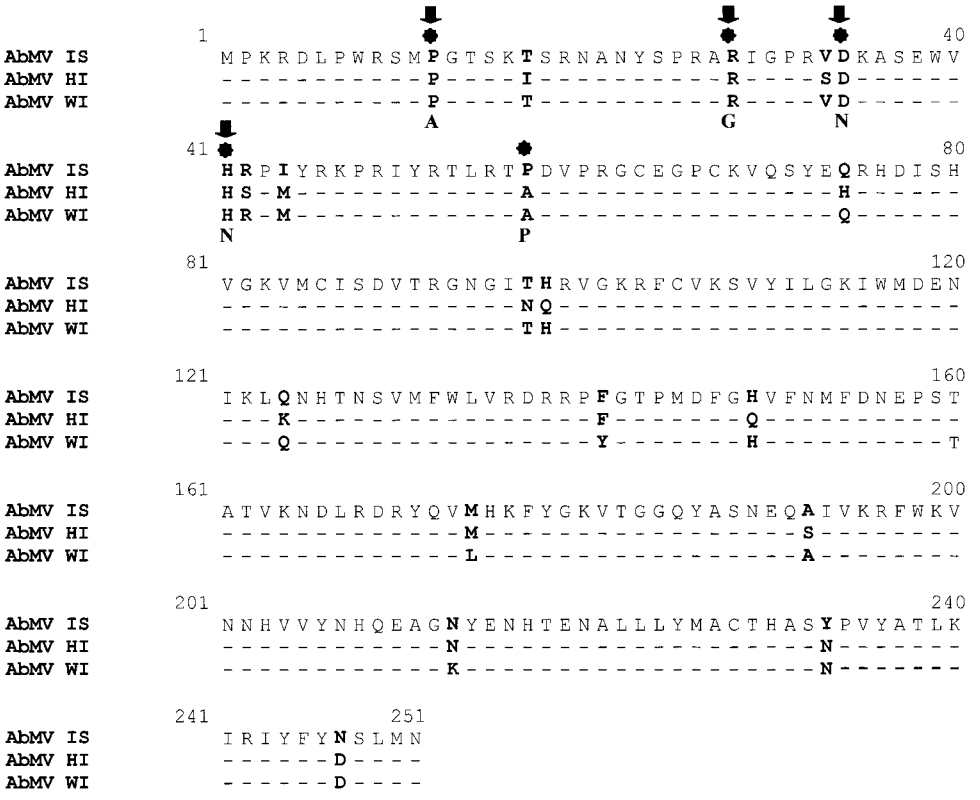


FIG. 6. Amino acid sequence of the CP of AbMV-Is and comparison with AbMV from Hawaii (AbMV-HI) and from the West Indies (AbMV-WI). Alignment of the predicted amino acid sequences from three whitefly nontransmissible AbMV isolates. The five amino acids suggested to be involved in transmission of bipartite geminiviruses are indicated by asterisks (Wu *et al.,* 1996). Amino acids that may be involved in the passage of geminiviruses from the whitefly gut into the hemolymph are indicated by arrows.

Clostridium thermocellum Hsp60/GroEL homolog (GenBank accession number P48212) and the glutamic acid to alanine substitution at position 388 is found in the *Haemophilus ducreyi* GroEL (GenBank accession number P31294), these substitutions seem to be of minor importance to the chaperone function of GroEL.

Cloning and sequencing of the CP of AbMV-Is

As a second step to study interactions between GroEL and begomoviral CP, we have cloned the CP of AbMV-Is. PCR primers were designed according to the sequence of the six N- and C-terminal amino acids of AbMV-WI (Frischmuth *et al.,* 1990). The CP gene of AbMV-Is was amplified from infected abutilon plants using primers AbMV-Is*Eco*RI and AbMV-Is*Xho*I, cloned and sequenced. The deduced amino acid sequence of AbMV-Is CP is shown in Fig. 6. The AbMV-Is CP shares 97 and 94% amino acid sequence identity, respectively, with the CP of AbMV isolates from the West Indies and from Hawaii (AbMV-HI, GenBank accession number U51137). The CPs of AbMV-Is and AbMV-WI share 23 of the 24 amino acids which differentiate AbMV-WI from the closely related transmissible SiGMV (Höfer *et al.,* 1997). The only exception is proline at position 57 in both AbMV-Is and SiGMV, instead of alanine in the CPs of AbMV-WI and AbMV-HI.

The CPs of TYLCV-Is and of AbMV-Is interact with *B. tabaci* GroEL

Our working hypothesis was that AbMV-Is is not transmitted because it is destroyed in the hemolymph immediately after crossing the gut, as a result of an impaired binding to GroEL. To test this assumption we have compared the ability of the CP of AbMV-Is and of TYLCV-Is to interact with GroEL in the yeast two-hybrid system. The two CP genes were fused in-frame to the LexA binding domain (BD) in the yeast expression vector pLexA, while the *B. tabaci* GroEL gene was fused in-frame to the B42 transcriptional activation domain (AD) in the yeast expression vector pB42AD. The GroEL plasmid (pB42AD-GroEL) together with either the TYLCV-Is or AbMV-Is CP-containing plasmids (pLexABD-TYCP and pLexABD-AbCP, respectively) were cointroduced into yeast containing the reporter plasmid p8op-lacZ. The plasmids were maintained in yeast using drop-out medium lacking uracil, tryptophan, and histidine.

To confirm that the CP and GroEL fusion proteins were indeed expressed in the yeast cells, extracts from cells cotransformed with pB42AD-GroEL and either pLexA-AbCP (cell line 6) or pLexA-TYCP (cell line 7) were subjected to gel electrophoresis, blotted, and probed with antiserum raised against LexA or GroEL. Figure 7 shows

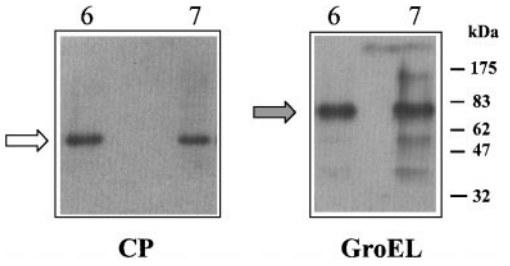


FIG. 7. Expression of CPs from TYLCV-Is, AbMV-Is, and GroEL in yeast. Expression of geminivirus CP and GroEL in yeast cells transfected with p8op-lacZ, pB42AD-GroEL, and either pLexABD-AbCP (cell line 6) or pLexABD-TYCP (cell line 7). Cell extracts were subjected to gel electrophoresis, blotted, and probed with antiserum raised against LexA (CP, left panel) or *Buchnera* GroEL (GroEL, right panel). The position of the ~55-kDa LexABD-Ab/TY/CP fusion protein is indicated by a white arrow, and that of the ~75-kDa B42AD-GroEL fusion protein is indicated by a gray arrow.

that in both cases, bands corresponding to the predicted size of the fusion proteins, ~55 kDa for LexABD-AbCP and LexABD-TYCP and ~75 kDa for B42AD-GroEL, were detected.

Yeast cells cotransformed with pB42AD-GroEL and either pLexA-TYCP or pLexA-AbCP were able to grow vigorously in the presence and in the absence of leucine (Fig. 8, lower panel, cell lines 6 and 7). When either the GroEL or the CP coding sequences were omitted from the pB42AD or pLexABD vectors, no significant growth was observed in medium lacking leucine, in contrast to extensive growth in the presence of leucine (Fig. 8, cell lines 3, 4, and 5). Throughout these experiments, all the yeast cell lines that grew in the absence of leucine expressed β-galactosidase activity and stained blue in a colony-lift assay (Fig. 8, lower panel, cell lines 6 and 7). A β-galactosidase activity of approximately 5 units was measured in both cell lines (Fig. 8, lower panel). These results demonstrated that in the yeast assay, *B. tabaci* GroEL interacted with the CP of both the transmissible TYLCV-Is and the nontransmissible AbMV-Is.

DISCUSSION

Although begomovirus circulative transmission is broadly accepted, the insect cellular factors that interact with the virion and permit its translocation are still poorly understood. Begomovirus particles ingested during feeding move into the digestive tract, enter the hemolymph probably at the level of the anterior midgut, reach the salivary glands, and are expelled during feeding (Markham *et al.,* 1994; Harris *et al.,* 1995; Hunter *et al.,* 1998; Rosell *et al.,* 1999). Transit in the hemolymph seems to be the most hazardous phase of the circulative path. The question of how viruses are protected in the hemolymph is fundamental to our understanding of the mechanism underlying circulative transmission. Insect endosymbiotic bacteria seem to play a crucial role in this process. The role of endosymbiotic GroEL chaperonins in permitting survival of viruses was first demonstrated in aphids. *Potato leaf roll virus* (PLRV) was dependent on a 63-kDa GroEL produced by the primary endosymbiont, a *Buchnera* sp., of the aphid *M. persicae* (van den Heuvel *et al.,* 1994). We have shown that the survival of TYLCV-Is in the hemolymph of *B. tabaci* is ensured by a similar strategy (Morin *et al.,* 1999), suggesting that viruses belonging to unrelated taxonomic groups have similarly taken advantage of insect endosymbiotic bacteria proteins to avoid degradation (Gibbs, 1999). We have previously presented circumstantial evidence strongly suggesting that a GroEL protein produced by *B. tabaci* coccoid bacteria interacts with TYLCV-Is particles in the insect hemolymph. Disturbing the putative interaction in the hemolymph by feeding whiteflies with an anti-GroEL antiserum led to an ~80% reduction in TYLCV-Is transmission. In the hemolymph of the treated whitefly TYLCV-Is DNA was reduced to amounts below the threshold of detection by Southern blot hybridization. In the present investigation, we have tested whether *B. tabaci* GroEL can interact with begomovirus CP *in vivo.* We have addressed the question of whether nontransmissibility of some begomoviruses can be related to a lack of interaction between the virus CP and GroEL. For our studies, we have used an isolate of AbMV from Israel as an example of a nontransmissible begomovirus.

Several studies have shown that AbMV isolates from different regions of the world are not transmitted by *B. tabaci* (Bedford *et al.,* 1994; Wu *et al.,* 1996; Höfer *et al.,* 1997). Similarly AbMV from Israel is ingested but not transmitted by *B. tabaci.* Although not transmitted, it is likely that at least some viral DNA is retained encapsidated in the insect since AbMV-Is DNA, presumed to be associated with the virus capsid, was detectable by immunocapture-PCR for up to 1 week after the insects had access to infected abutilon plants. The site(s) where the various AbMV isolates is blocked in the whitefly has not been identified. We have used PCR to show that AbMV-Is DNA was never detected beyond the midgut, even after ingestion periods of 4 days. The long-term persistence of AbMV in the insect digestive tract was not the result of its protection by insect GroEL, since this protein was undetectable in the midgut at the time it was evident in the hemolymph. Whether the reason for not detecting AbMV-Is beyond the gut was the result of the virus's inability to cross the gut/hemolymph barrier or to its rapid destruction immediately after entering the hemolymph was not clear. However, since we have previously suggested that binding between *B. tabaci* GroEL and TYLCV-Is particles is crucial to the virus survival in the insect hemolymph (Morin *et al.,* 1999), we have asked if *B. tabaci* GroEL is able to interact with a begomovirus CP *in vivo.* The yeast two-hybrid system was used to compare the interaction between GroEL and the CPs of AbMV-Is and TYLCV-Is.

	Transfected yeast cells						
Plasmids	**1**	**2**	**3**	**4**	**5**	**6**	**7**
p8op-LacZ	+	+	+	+	+	+	+
p42AD		+	+	+			
p42AD-GroEL					+	+	+
pLexABD		+			+		
pLexABD-CP-AbMV			+			+	
pLexABD-CP-TYLCV				+			+
Growth on medium without Leucine	**no**	**no**	**no**	**no**	**no**	**yes**	**yes**
β-galactosidase activity	**no**	**no**	**no**	**no**	**no**	**yes**	**yes**

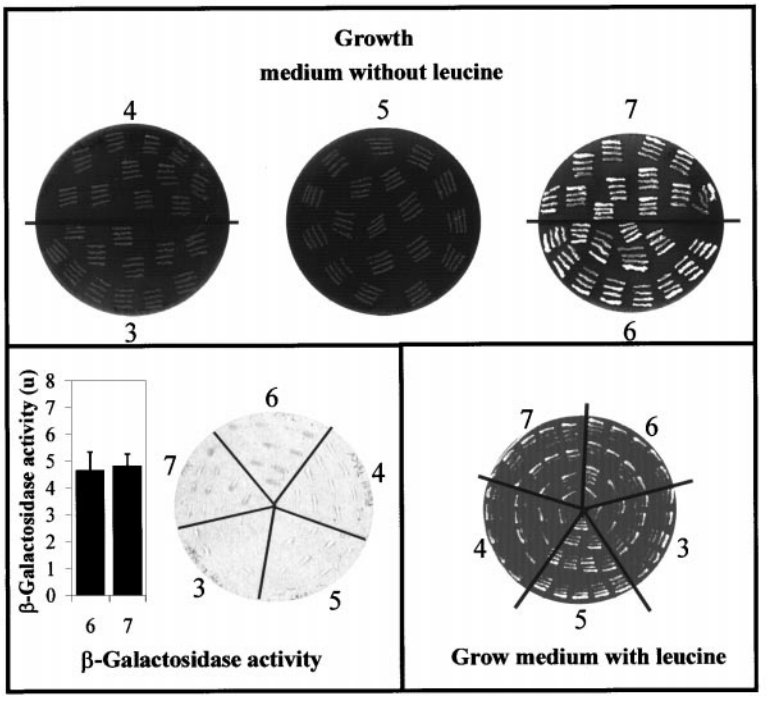


FIG. 8. Interaction of CPs from TYLCV-Is and AbMV-Is with *B. tabaci* GroEL in yeast. Upper panel: Growth of yeast cell lines, numbered 1 to 7, transfected with various plasmids (+), and their β-galactosidase activity in medium lacking leucine. Lower panel: Growth of transfected yeast cell lines (numbers correspond to those in the upper panel) in petri dishes with medium lacking or containing leucine, and β-galactosidase activity of the yeast cell lines grown in medium lacking leucine. β-Galactosidase activity was quantified using duplicate samples from two individual colonies of each cell line. The activity of lines 6 and 7 was corrected for background activity of lines 3, 4, and 5.

In a first step, we cloned and sequenced a GroEL gene homolog from the *B. tabaci* B biotype. The first 30 N-terminal amino acids encoded by the GroEL gene were identical to those of the GroEL protein we sequenced previously (Morin *et al.,* 1999), indicating that the isolated gene encodes the previously isolated protein. The reason we emphasize this point is that analyses of 16S rDNA has indicated that *B. tabaci,* in contrast to the greenhouse whitefly *T. vaporariorum* and the ash whitefly *Siphoninus philyreae,* harbors primary and secondary endosymbionts, both of which are placed within the γ-subdivision of the *Proteobacteria.* The primary endosymbiont was placed between the γ-2 or γ-3 subgroups (Clark *et al.,* 1992), while the secondary endosymbiont was classified as a member of the *Enterobacteriaceae* group (Clark *et al.,* 1992; Baumann *et al.,* 1993). During the cloning process we were aware that both endosymbionts may harbor a GroEL gene homolog, and therefore tried to avoid amplifying a chimeric gene. We have two reasons to believe we have overcome this problem: (1) During PCR-based cloning, the 5′ primers were always designed according to the sequence obtained from the previous amplified fragments, starting with primer F31, which was based on the protein N-terminal sequence. (2) The sequence of the full-length gene (amplified using primers F-86 and OUT) was identical to that of the over-

lapping PCR fragments. The amplified gene probably originates from the genome of the coccoid endosymbiont, since only the cytoplasm of this bacteria was significantly labeled in our previous immunogold-labeling experiment (Morin *et al.,* 1999).

In a second step we have cloned the CP of AbMV-Is. The origin of the Israeli isolate of AbMV is unlikely to be local. Most probably it was imported with ornamentals and spread by local commercial nurseries. The high degree of homology between the CP of AbMV isolates from several regions in the world indicates that these isolates most likely have a common origin.

We have expressed the cloned *B. tabaci* GroEL as a fusion protein in the yeast expression vector pB42AD. The CP ORFs of the whitefly transmissible TYLCV-Is and the nontransmissible AbMV-Is were also cloned and expressed as fusion proteins. The two-hybrid assay clearly demonstrated that, in this system, GroEL interacted with the CP of AbMV-Is as it did with that of TYLCV-Is. These results indicate that rapid degradation in the hemolymph resulting from an incorrect GroEL–CP interaction is probably not the mechanism responsible for the nontransmissibility of AbMV-Is. On the other hand, it is possible that the yeast binding assay does not reflect the situation in the gut. In yeast cells, monomers of CP interact with monomers of GroEL (both as fusion proteins) in a one-to-one stoichiometric ratio, while in the hemolymph it is expected that one or more virions interact with one or more multimeric GroEL complexes. Interaction between monomers may not necessarily reflect the interaction between multimeric complexes.

The results of the two-hybrid assay, showing binding of AbMV CP to GroEL, strongly suggest that AbMV-Is is blocked at the level of the gut wall barrier and seemingly does not reach the hemolymph. The involvement of the gut epithelia in the regulation of virus acquisition by the insect has been reported in several insect-virus systems. Ultrastructural and serological analyses have shown that a midgut barrier is the cause of the inability of adult western flower thrips *Frankliniella occidentalis* to acquire *Tomato spotted wilt virus* (Ullman *et al.,* 1992). In aphids, recognition of some luteoviruses occurs at the hindgut membrane (Gildow, 1987, 1993). In whiteflies, DNA of the transmissible begomovirus SqLCV could be detected in whole body homogenates and honeydew but not in the hemolymph or saliva of the nonvector whitefly *T. vaporariorum,* suggesting that the gut epithelia of the nonvector whitefly does not permit passage of SqLCV particles into the hemocoel (Rosell *et al.,* 1999). On the basis of these data and on the results presented in this study, we postulate that *B. tabaci* gut epithelial cells serve as the first barrier that begomoviruses have to cross to be transmitted. It is likely that AbMV-Is has lost the ability to bind to receptors within the *B. tabaci* digestive tract, which facilitate the passage of virions into the hemocoel, probably because it has been maintained and propagated in abutilon cuttings.

The specificity of geminivirus transmission may result from an intimate relationship between surface epitopes of the CP and certain insect receptors. There is no evidence for the involvement of other virus-coded proteins in insect transmission, as for the aphid transmission factors of caulimoviruses and potyviruses (Pirone and Blanc, 1996) or the read-through domain of luteoviruses (Brault *et al.,* 1995). The amino acids of the AbMV CP that are implicated in nontransmission are not known. Comparing the CP of AbMV-WI with that of the closely related transmissible SiGMV has shown 24 amino acid differences (Höfer *et al.,* 1997). Aligning the CP amino acid sequences of two AbMV isolates (West India and Hawaii) with that of 19 transmissible bipartite begomoviruses has shown that only five of the 24 amino acids are identical in the two AbMV strains, but different from the consensus sequence of the transmissible viruses, that is, proline at position 12, arginine at position 28, aspartate at position 34, histidine at position 41, and alanine at position 57 (Wu *et al.,* 1996). In this study, the alignment of the CP of AbMV-WI and AbMV-HI with that of AbMV-Is has indicated that the alanine-proline substitution at position 57 is probably not implicated in geminivirus transmission. We therefore postulate that some or all of the four amino acids at positions 12, 28, 34, and 41 are involved in the ability of bipartite geminiviruses to penetrate the gut epithelia.

It has been shown that luteoviruses bind *in vitro* to the N-terminal (amino acids 1–121) and C-terminal (amino acids 409–474) regions of the GroEL equatorial domain. It was suggested that these domains constitute alternative binding sites for large multimeric proteins unable to overcome the size limitation imposed by the GroEL central cavity (Hogenhout *et al.,* 1998). Comparison of these regions with the *B. tabaci* GroEL homolog domains shows extremely high homology (~96%) in the N-terminal region and moderately high homology (~87%) in the C-terminal region. Therefore the N-terminal domain may constitute a conserved binding site of macromolecules. If this is the case, it will be interesting to find out whether the affinity between begomoviral particles and the GroEL subunit is confined to this region of the protein.

MATERIALS AND METHODS

Maintenance of virus cultures, whiteflies, and plants

Bemisia tabaci of the B biotype (Cohen, 1993) was reared on cotton plants (*Gossypium hirsutum,* cv. Akala) grown in insect-proof wooden cages at 24–27°C. Cultures of an Israeli isolate of TYLCV-Is (Navot *et al.,* 1991) were maintained in tomato plants (*Lycopersicon esculentum,* cv. Daniella). Cultures of the Israeli isolate of AbMV-Is were maintained by cuttings in *Abutilon sellovianum* plants. All insects used were females.

Access of adult insects to AbMV-Is-infected abutilon and TYLCV-Is-infected tomato plants

All experiments were conducted in insect-proof wooden cages kept at 24–27°C in an insect-proof growth chamber. Five to 8 days after emergence, the insects were caged with the youngest true leaf from the infected tomato or abutilon plant for the time periods indicated in the experiments.

Amplification of AbMV-Is and TYLCV-Is DNA using organs dissected from whiteflies as substrate for PCR

After various access periods on infected plants, the insects were collected by aspiration and were exposed to acetone vapors to arrest movement. The insects were dissected on a glass slide under a binocular microscope (×40). Dissections were performed in insect physiological saline (IPS: 4.5 g NaCl, 0.1 g KCl, 0.1 g $CaCl_2$, 0.1 g $MgCl_2$, 0.1 g $NaHCO_3$, 2.0 g glucose, per liter) with or without 1% toluidine blue, on a glass slide as described (Bandla *et al.,* 1998). The isolated tissues were deposited into a PCR test tube containing 3 μl sterile double-distilled water (ddw). To dissect the midgut, the abdomen was separated from the thorax at the connection between them. Its content was expelled in a drop of IPS (without toluidine blue) by pushing gently on the abdomen. The midgut was isolated, cleaned from other tissues, flushed several times with ddw, and collected using a fine metallic thread fixed on a wooden handle. To isolate the salivary glands, the prothorax was separated from the mesothorax and abdomen. The glands were kept for 2–5 min in IPS–toluidine blue until the primary salivary glands absorbed the dye, allowing identification and dissection. The salivary glands were flushed several times with ddw and collected. Hemolymph was extruded and collected with a glass micropipette as described (Morin *et al.,* 1999). The insect organs, without any further treatment, were subjected to PCR.

Whitefly DNA and isolated organs were subjected to PCR (20-μl reaction) as described (Ghanim *et al.,* 1998). The cycling protocol was as follows: initial denaturation for 3 min at 95°C, annealing of primers for 1 min at 55°C, extension for 2 min at 72°C, and denaturation for 1 min at 94°C; subsequent cycles were: 1 min at 55°C, 2 min at 72°C, and 1 min at 94°C; after 30 cycles, the PCR products were subjected to electrophoresis in a 1% agarose gel, blotted, and hybridized with full-length cloned geminiviral DNA (Ghanim *et al.,* 1998) when specified. Autoradiography was for 1–8 h using X-ray films (Fuji).

The PCR primers used were derived from the sequence of AbMV-WI DNA-A and DNA-B (Frischmuth *et al.,* 1990) and TYLCV-Is (Navot *et al.,* 1991). For AbMV-Is: an ~760-bp fragment was amplified from DNA-A using primers AbAV356 (position 356–379, virion strand, 5′-CAAAATGCCTAAGCGCGATCTCCC-3′) and AbAC1117 (position 1117–1096, complementary strand, 5′-TTTATTAATTCATGAGCGAATC-3′), an ~660-bp fragment was amplified from DNA-B using primers AbBV659 (position 659–682, virion strand, 5′-CGACCTTCAAATACGAGCAAGCCC-3′) and AbBC1318 (position 1318–1292, complementary strand, 5′-CTTAACCAATATAGTCAAGGTCAAACG-3′). For TYLCV-Is: an ~410-bp fragment was amplified using the primers TYV61 (position 61–80, virion strand, 5′-ATACTTGGACACCTAATGGC-3′) and TYC473 (position 473–457, complementary strand, 5′-AGTCACGGGCCCTTACA-3′). Oligonucleotides were purchased from Biotechnology General (Rehovot, Israel).

Use of immunocapture-PCR to detect viral DNA associated with AbMV CP in insects and in plants

AbMV viral DNA, presumed to be associated with the virus coat protein (CP), was detected by immunocapture-PCR (Jacobi *et al.,* 1998), using an antibody raised against TGMV virions. The buffers used for immunocapture-PCR are as described by the manufacturer (Bioreba, Reinach, Switzerland). PCR tubes were filled with 200 μl of antiserum (1:500 diluted in coating buffer), incubated for 3 h at 37°C and washed five times for 5 min with 200 μl washing buffer. Whitefly (20 insects) or plant homogenates (~50 mg) in 200 μl of extraction buffer were incubated in the coated PCR tubes for 18 h at 4°C. The tubes were then washed five times for 5 min with 200 μl washing buffer and dried. PCR-amplification of the viral DNA from the virions bound to the antibody-coated tubes was performed using the AbMV DNA-A primer pair AbAV356 and AbAC1117, as described earlier.

Polyacrylamide gel electrophoresis (PAGE) of proteins, blotting, and immunodetection of GroEL

Hemolymph samples and midgut collected from 30 whiteflies were incubated in sample buffer (0.125 M Tris–HCl, pH 8.8, 20% glycerol, 0.001% bromphenol blue) and 40 μl was subjected to nondenaturing PAGE (5% acrylamide, 0.13% bisacrylamide, 0.375 M Tris–HCl, pH 8.8) at 80 V (10–15 mA) for 4 h (Mini-Protein II Cell; Bio-Rad, Hercules, CA). Partially purified native GroEL (Morin *et al.,* 1999) was used as marker. In addition, the hemolymph and native GroEL samples were homogenized in 60 μl Laemmli buffer and 40 μl were subjected to 10% SDS–PAGE (Laemmli, 1970). After electrophoresis, proteins were electroblotted and GroEL was immunodetected using antibodies raised against *Buchnera* GroEL from *M. persicae* (van den Heuvel *et al.,* 1997) as described (Morin *et al.,* 1999).

Cloning and sequencing of the *B. tabaci* GroEL gene

Total DNA from approximately 1000 whiteflies (4 to 7 days after emergence) was isolated using the QIAamp tissue kit protocol for insects (Qiagen, Chatsworth, CA). PCR fragments containing the *B. tabaci* GroEL sequence were generated using the primers listed in Fig. 4. PCR

amplification was performed in a final volume of 50 μl 10 mM Tris–HCl (pH 8.3) containing 1 μg of insect DNA, 0.4 μM of each primer, 0.1 mM of each of four deoxynucleotide triphosphates (dNTPs), 3 mM $MgCl_2$, 50 mM KCl, and 2.5 units of *Taq* polymerase (Boehringer Mannheim, Indianapolis, IN). The cycling protocol was as follows: initial denaturation for 3 min at 95°C, annealing of primers for 1 min at 60°C, extension for 2 min at 72°C; 5 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C; 10 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C; 30 cycles of 1 min at 94°C, 1 min at 50°C, 2 min at 72°C; 30 min at 72°C. PCR products were analyzed on agarose gels. PCR products were purified from gels using the QIAquick gel extraction kit (Qiagen). The full-length *B. tabaci* GroEL gene and its flanking regions were amplified by *Pfu* polymerase (Promega, Madison, WI). The resulting ~1900-bp PCR product was cloned using the pGEM-T Easy vector system (Promega). The resulting plasmid was called pGEM-GroEL-B. The sequence was established by the DNA Sequencing Unit of the Weizmann Institute of Science (Rehovot, Israel).

Cloning and sequencing the CP ORFs of AbMV-Is and TYLCV-Is

Fragments encompassing the full-length CP ORFs of AbMV-Is (AbCP) and TYLCV-Is (TYCP) were PCR-amplified from DNA purified from infected plants as described (Ghanim *et al.,* 1998). For AbMV-Is the primers AbMV-Is*Eco*RI (5′-CGGAATTCATGCCTAAGCGCGATCTC-3′) and AbMV-Is*Xho*I (5′-CCGCTCGAGTTAATTCATGAGCGAATC-3′) were used to introduce *Eco*RI and *Xho*I restriction sites (underlined) at the 5′ and 3′ termini of the CP ORF, respectively. For TYLCV-Is we followed the same strategy, this time using the primers TY*Eco*RI (5′-CGGAATTCATGTCGAAGCGACCAGGC-3′) and TY*Xho*I (5′-CCGCTCGAGATTTGATATTGAATCATA-3′). The PCR products were cloned using the pGEM-T Easy vector system (Promega) to give pGEM-AbCP and pGEM-TYCP, respectively. The cloned genes were sequenced.

Cloning the TYLCV-Is and AbMV-Is CP ORFs, and the *B. tabaci* GroEL gene in yeast plasmids

The two CP ORFs were released from pGEM-AbCP and pGEM-TYCP by digestion with *Eco*RI and *Xho*I. They were cloned in-frame with the LexA binding domain, into the same restriction sites of the yeast plasmid pLexA that contained a *HIS3* marker (Clontech, Palo Alto, CA). The recombinant plasmids were named pLexA-AbCP and pLexA-TYCP.

The full-length GroEL gene was PCR-amplified from plasmid pGEM-GroEL-B using the primers OUT (Fig. 4) and ECOEL (5′-CGGAATTCATGGCAGCTAAAGACTTAAAA-3′), which introduced an *Eco*RI site at the 5′-terminus of the GroEL gene. The PCR product was cloned using the pGEM-T Easy vector system (Promega) to give plasmid pGEM-ECoGroEL. The *Eco*RI fragment encompassing the GroEL gene was released from pGEM-ECoGroEL. It was cloned in-frame with the B42 activation domain into the *Eco*RI site of the yeast plasmid pB42AD, which contained a *TRP1* marker (Clontech). The recombinant plasmid was named pB42AD-GroEL.

Expression of TYLCV-Is and AbMV-Is CP in the yeast two-hybrid system and their binding to *B. tabaci* GroEL

Plasmid pB42AD-GroEL together with plasmid pLexA-AbCP or pLexA-TYCP were introduced into the yeast strain EGY48 containing the reporter plasmid p8op-lacZ (Clontech), using the lithium acetate-mediated method (Becker and Lundblad, 1997). In the EGY48 strain, the upstream activating sequences of the chromosomal *LEU2* gene, required in the leucine (Leu) biosynthetic pathway, are replaced with LexA operators (DNA-binding sites). Plasmid p8op-lacZ contains a LexA operator-lacZ fusion gene. In the first plating, transformed yeast cells were plated on complete minimal (CM) medium (Treco and Lundblad, 1997) lacking uracil (Ura), histidine (His), and tryptophan (Trp), with 2% glucose as a sugar source. In the second plating, which selects for yeast that contain interacting proteins, selected primary transformants were plated on CM medium lacking Ura, His, Trp, and Leu with 2% galactose/1% raffinose as the sugar source. β-Galactosidase activity was monitored by colony lift and liquid assays using X-gal and ONPG as substrates as described by Reynolds and Lundblad (1997). One unit of β-galactosidase is defined as the amount of enzyme necessary to hydrolyze 1 μmol of ONPG to *o*-nitrophenol and D-galactose per min per cell.

To confirm that the fusion proteins are synthesized properly, a yeast cell crude lysate was prepared (Golemis *et al.,* 1997), fractionated by 10% SDS–PAGE (Laemmli, 1970), and electroblotted (Morin *et al.,* 1999). Fusion proteins expressed from pLexA-AbCP or pLexA-TYCP were detected using LexA monoclonal antibody (Clontech). Fusion proteins expressed from pB42AD-GroEL were immunodetected using antibodies raised against *Buchnera* GroEL from *M. persicae* (van den Heuvel *et al.,* 1997).

Yeast growth in the absence/presence of Leu was evaluated by incubating the cells for 48 h at 28°C in a Leu-containing medium. The cells were then streaked (four ~1-cm rows per cell line) on agar petri dishes containing medium with and without Leu. The plates were scanned after an additional 48-h incubation. To test the β-galactosidase activity, the yeast colonies grown on Leu-containing dishes were lifted on a nylon membrane. After three rounds of freezing in liquid nitrogen and thawing, the membrane was placed on Whatmann 3M paper soaked with buffer Z containing 1 mg/ml X-gal (Golemis *et al.,* 1997). A purple color appeared within 20 min.

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